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TITLE: The Role of the Rab Coupling Protein in ErbB2-Driven Mammary Tumorigenesis and Metastasis

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14. ABSTRACT

During the Year-2 we focused in our understanding of the role of the Rab Coupling Protein (RCP) in ErbB2-mediated lung metastasis formation. To accomplish this, we have derived both proficient and deficient ErbB2 stable expressed cell lines and then performed orthotopic mammary fat pad and tail vein cell injection using the classic immunodeficient NCr athymic mouse model as syngenic recipient. Consistent with our previous in vivo data, the overexpression of RCP led into drastically decrease the number of lung. In contrast, the depletion of RCP using RNA interference approach significantly increase the number of lung metastases.

15. SUBJECT TERMS
Breast Cancer, Oncogene-Induced Tumorigenesis, Metastasis Formation, Receptor Tyrosine Kinase, Her/ErbB2 signaling, Rab Coupling Protein/Rab11FIP1/RCP, Epithelial Mesenchymal Transition, Cell junctions, Cell Proliferation

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INTRODUCTION

ErbB2 is a member of the EGFR family of receptor tyrosine kinases (RTKs). This family is comprised of four closely related type 1 RTKs that include EGFR, ErbB2 (Neu, HER2), ErbB3 (HER3), and ErbB4 (HER4). The importance of ErbB2 in primary human breast cancer is highlighted by the fact that 20-30% of human breast cancers express elevated levels of ErbB2 due to the genomic amplification of the *erbB2* proto-oncogene. The potent transforming potential of ErbB2 in the mammary epithelium is thought to be due to its capacity to couple with a number of Src homology 2 (SH2) and phosphotyrosine binding (PTB) domain containing signaling molecules. Following activation of the intrinsic tyrosine kinase activity of ErbB2, specific phosphotyrosine residues within ErbB2 provide binding sites for a variety of SH2 or PTB domain-containing signaling molecules including ShcA, Grb2, Src family kinases and p85 subunit of the phosphatidylinositol 3' kinase (PI-3K). Although ErbB2 coupled signaling has been extensively studied, the molecular mechanism involved in trafficking ErbB2 Receptor Tyrosine Kinases (RTK) from the cell surface to different sub-cellular compartments is poorly understood. In this regard, the Rab family of GTPases are thought to play a critical role in the trafficking of a number of RTKs For example, the recycling endosomes that are involved in recycling activated ErbB2 to the plasma membrane are enriched in both the Rab4 and Rab11 GTPases. A number of Rab11/25-interacting proteins, also known as the Rab11-FIP/Rip/RCP family, are thought to be important Rab effector molecules. Rab Coupling Protein (RCP), which acts as an effector for Rab11 and Rab25, mediates recycling of the EGFR/$\alpha5\beta1$-integrin complex to plasma membrane, a pre-requisite for cell invasion. Additionally, RCP contributes to recycling of phagosomes in macrophages and has also been implicated as a critical regulator of cell polarity in epithelial cell lines. Nonetheless, the biological and molecular function of RCP in epithelial cell junction dynamics and tumorigenesis is poorly understood.

In addition to its role in regulating epithelial polarity, there is an increasing body of evidence implicating RCP in cancer progression. For example, RCP-mediated recycling of the integrin $\alpha5\beta1$ is activated by expression of mutant forms of p53 resulting in enhanced cancer cell invasion. In 10% of human breast cancers amplification and elevated expression of RCP is associated with poor clinical outcome. Moreover elevated expression of RCP is correlated with a poor clinical outcome in squamous cell carcinomas of the head and neck. Consistent with view that Rab25/RCP signaling promotes tumor progression, elevated expression of Rab25 in breast and ovarian cancer resulted in enhanced tumorigenic growth. However, there is also evidence that Rab25/RCP signaling can suppress tumor growth. For example, in triple negative breast cancer, esophageal, and colon tumor Rab25 expression can suppress tumor growth. These observations argue that ability of Rab25/RCP signaling to promote or suppress tumor growth is highly dependent on the tissue context.

Given that breast cancer is comprised of multiple biologically distinct subtypes, the role of Rab25/RCP signaling in ErbB2 tumor progression is unclear. To directly investigate the role of RCP in ErbB2 mammary tumor progression, we evaluated the consequences of modulating RCP levels in the mammary epithelium in a number of mouse models of ErbB2 mammary tumor progression. Using a MMTV activated ErbB2 strain (NDL model), we show that inducible expression of RCP results in a significant delay in mammary tumor onset. Conversely, targeted deletion of RCP in the mammary epithelium of MTTV/activated ErbB2 IRES Cre transgenic mouse model (NIC model) resulted in accelerated tumor onset that was further associated with an enhanced metastatic potential. The ability of RCP to modulate tumor progression in both these ErbB2 models of breast cancer reflected the key role of RCP in the endocytic trafficking of both E-Cadherin and ZONAB junctional complexes that further impact on Cdk4-mediated cell proliferation. In addition, to regulating trafficking of these key cell junctional proteins, we further demonstrate that RCP is also involved in the down-regulation of ErbB2 to lysomal compartment. Consistent with a role of RCP as critical negative regulator of ErbB2 mediated signaling, we further show that the expression levels ErbB2 and RCP are inversely correlated in human breast cancers. Together these observations indicate that, at least in the context of ErbB2 driven breast cancer, RCP acts to suppress tumor cell proliferation and metastatic potential.

KEYWORDS

Breast Cancer, Oncogene-Induced Tumorigenesis, Metastasis Formation, Receptor Tyrosine Kinase, Her/ErbB2 signaling, Rab Coupling Protein/Rab11FIP1/RCP, Epithelial Mesenchymal Transition, Cell junctions and Cell Proliferation.

ACCOMPLISHMENTS

The PI is reminded that the recipient organization is required to obtain prior written approval from the awarding agency Grants Officer whenever there are significant changes in the project or its direction.

3.1 Major goals of the project for Year-2

During the Year-2, we studied the role of RCP in ErbB2-driven metastasis program using in vivo transgenic and syngenic breast cancer mouse models. To complete this, we used both proficient and deficient RCP models which consist in RCP-overexpressed and RCP-deleted cell lines (RCP/ErbB2 model), respectively. The metastatic fate was evaluated by orthotopically injecting the RCP/ErbB2 cell lines through mammary fat pad and tail vein injection techniques. This aim is subdivided as below. All SOW objectives and tasks are cited as suggested in the original SOW.

SOW-Objective 2. To characterize mammary tumour progression as well as metastasis formation in RCP-proficient and RCP-deficient mouse model.

SOW-Task 1.*Attain ethical approval from ACURO for the use of mice (Months 2-3)*
Evolution. *Completion: 100%.*

To follow the ethical approval from ACURO guidelines our Molecular Oncology Group, currently holds an approved **Animal Use Protocol # 2008-5518** with McGill University following the high standards established by the Canadian Council on Animal Care.

SOW-Objective-1. *To elucidate the role of RCP in ErbB2 endocytosis as well as in Histone-3 methylation to control oncogenic activity of ErbB2 receptor.*

SOW-Task 4. *Determine the effects of RCP-proficiency and deficiency on pro-tumorigenic and pro-metastatic properties of ErbB2-driven tumor cells. (Months 6-11).*
Evolution: *Completion: 100%.*

4a. *Transplant RCP-ErbB2-tumor cell models (TM15, SKBR3 and BT474) pre-treated or not with the antibody 7.16.4 into the mammary fat pads of syngeneic recipients and allow growing until they become physically palpable.*

Regarding this task 4a, we were able to transplant TM15 and SKBR3 and BT474 cell lines in syngenic mice. This Task-4a was conducted in our Mouse facility. A subset of cells were pre-treated with a murine version of human anti-Her2 monoclonal antibody *Herceptin* called 7.16.4. Our data indicates that overexpression of RCP as well as depletion of RCP does not significantly modulates mammary tumor growth (Fig. 1a). However, the anti-ErbB2 7.16.4 antibody treatment led into significantly delayed tumor growth (Fig. 1a). We next assessed human breast cancer cell lines to similar treatment. To bypass clearance of human cell transplant, we mixed SKBR3 and BT474 cells with Matrigel prior injection. Similarly, the overexpression of RCP in ErbB2-positive human breast cancer cell lines exhibit no alteration in tumor growth kinetics. However, the subset of RCP/ErbB2 cells treated with *Herceptin* antibody significantly decelerated tumor growth rate in all cell conditions (*data not shown*).

4b. *Administer in xenograft mice (Obj.2 Task 4a) either antibody 7.16.4 (10 mg per kg−1) or vehicle control (IgG) intraperitoneally once per week. Estimate 5 mice per group: Empty vector, RCP-GFP and shRCP (3 experimental groups) = 15 mice per experiment. Treated with IgG or antibody. Total of 30 mice per cell lines x 3 types of cell lines = 90 mice.*

RCP/ErbB2 cell lines were subjected to mammary fat pad injection and monitoring of tumor growth. Accordingly with palpable-tumor volume chart, as soon as tumors were palpable, subset of mice were treated with IgG or 7.16.4 antibody twice a week. The overexpression of RCP combine to 7.16.4 treatment drastically abrogates tumor growth compared

to control IgG condition (Fig. 2a). However, anti-ErbB2 therapy is sufficient to decelerate the growth kinetic of RCP-depleted cells (Fig. 1b).

4c. *Tail vein injection of RCP-ErbB2-tumor cell models as in Obj.2 Task-2b, pre-treated or not with Herceptin in immunocompromised mice. Estimate 5 mice per group: Empty vector, RCP-GFP and shRCP (3 experimental groups) = 15 mice per experiment. Treated with IgG or antibody. Total of 30 mice per cell lines x 3 types of cell lines = 90 mice. Animals will be sacrificed 4 weeks after cell injection. Lungs are collected and GFP-cells are counted by flow-cytometry.*

Erratum: we used 7.16.4 instead of Herceptin for cell species purpose as explain as above

Our data indicates that RCP acts as a major negative regulator of ErbB2-mediated metastasis program. Treatment of control empty vector cells with 7.16.4 decrease lung metastases number. Interestingly, the overexpression of RCP (IgG condition) drastically suppress productivity of lung metastases (Fig. 2). Similarly, RCP-overexpressed cells treated with 7.16.4 harbor no lung metastases (Fig. 2). In contrast, the depletion of RCP (IgG condition) increases the number of lung metastases (Fig. 2). RCP-depleted cells treated with 7.16.4 antibody demonstrated similar number of lung metastases as in control empty vector cell condition (Fig. 2). On the other hand, we examine our lung specimens by flow cytometry and observe a signal associate with number of lung metastases (data not shown). However, the detection of GFP indicates some background signal from lung parenchyma matrix decreasing the stringency of our data analysis. For statistical purpose, we choose the number of lung metastases as output reading for lung metastasis monitoring. Altogether, our finding indicates that RCP is a potent negative regulator of metastatic cascade in ErbB2-positive breast cancer.

4d. *Quantification of data analysis. Lung metastasis results will be expressed as the number of lung mets as well as the occupied area (cm^2) from hematoxylin staining. ANOVA statistical analysis will be assessed between different groups.*

Quantification was performed as indicated, see Fig. 3.

SOW-Objective-2. Characterize mammary tumour progression as well as metastasis formation in RCP-proficient and RCP-deficient mouse model.

SOW-Task 5. Generate and characterize the inducible RCP knockout mouse model (Years 1-3):
Evolution: *Completion 100%.*

5b. *Generate inducible-RCPKO mice: cross Tetracycline operator-inducible ErbB2-IRES-Cre (NIC) with floxed RCP mice. Obtain RCP wild-type-NIC, RCP-heterozygote-NIC and RCP-homozygote-NIC. Cross a second cohort of floxed RCP with MMTV-Cre mice models for mammary gland outgrowth experiments.*

To determine whether mammary epithelial disruption of RCP function can affect normal mammary branching development, we breeded a MMTV-Cre mouse strain to a conditional RCP strain (see previous report) Specific both allele deletion of RCP in mammary epithelium seems to decrease the number of terminal end buds (see previous report).

5c. Cross MTB and floxed RCP mice. Obtain heterozygotes MTB and floxed RCP mice.

Erratum: Here is not MTB but MMTV-Cre.
As previously showed the Cre recombinase well excised RCP allele (See related figure as in last Report Year-1).

5d. *Cross TetO-NIC-RCP with RCP-MTB mice. Estimate 20 mice per group: wt and floxed RCP with doxycyclin and 10 mice per group: wt and floxed RCP without doxycycline = 60 mice in total.*

Erratum: Here is NIC/RCP is not a TetO system and no breeding with RCP-MTB. The tumor onset experiment NIC/RCPKO includes: NIC/RCP-WT (n=20 mice), NIC/RCP-het (n=20 mice) and NIC/RCP-homo (n=20 mice) for a total of 60 mice

Our data indicates that ablation of both RCP allele significantly accelerates tumor onset (Fig. 3).

5e. Mammary gland outgrowth experiment using MMTV-IRES-Cre-floxed-RCP and MMTV-floxed-RCP. Mammary glands extraction, H&E

Hematoxylin staining of mammary wholemount showed that excision of both RCP allele seems to decrease the number of terminal end buds but not alter the mammary branching as well as epithelium content (see previous report).

SOW-Task 6. Characterize the RCP-proficient mouse model (Years1-2)
Evolution: *Completion 100%.*

6c. *All data analysis will be represented as in Task-5, objective 5f.*

Data relative to Task 6c were included in previous report. See Supplemental figure 2 for Reminder purpose.

6d. *Monitor tumor-onset of RCP-proficient RCP-MTB NDL mice. Lung was collected for metastasis analysis. Estimate 20 mice per group = 40 mice in total*

Given the minimal impact on normal mammary gland outgrowth, we next evaluated whether epithelial expression of RCP could influence ErbB2 mammary tumor progression. To accomplish this we crossed into the inducible RCP transgene combination into mice carrying a NDL (activated ErbB2 transgene and induced RCP expression by oral administration of doxycycline. By contrast to control group of female NDL mice that were treated with doxycycline, mammary epithelial specific induction of RCP in this ErbB2 tumor model led to a significant decrease in tumor onset (Fig.3B. Interestingly, expression of the RCP transgene in the induced cohort could be further subdivided into two groups that expressed different levels of the RCP transgene (Fig. 3B). Amongst the cohort that expressed elevated levels of RCP as measured by immunoblot analyses the delay in tumor onset was more profound than those female mice that expressed lower levels of RCP (Fig.3C. These observations indicate that delay in tumor onset was directly correlated with RCP levels.

6e. Quantification of tumorigenesis and metastasis will be represented as in Task-5, objective 5h.

Quantification of tumorigenesis is indicated at figure 3.

Lung metastasis study—To accomplish this, we enumerated both the penetrance of the metastatic phenotype and number of metastatic lesions in inducible RCP ErbB2 model (Fig.4 left panel). Although induction of RCP expression in the NDLtumor model did not significantly alter either the penetrance or the number of metastatic foci (Fig. 4 middle panel), we noted that RCP proficient lesion were primarily intravascular in nature (Fig.4 right panel). This vasculature phenotype was determine by H&E staining.

SOW-Task 7. *Role of RCP in intravasation and lodging of ErbB2 derived tumor (Years 1-2)*
Evolution: *Completion 55%*

7a. *Blood was collected from mice subjected for tumor onset experiment as in Obj.2 Task 2-1c at the time of sacrifice and GFP-positive tumoral cells were counted by flow cytometry. Estimate 20 mice per group = 40 mice in total*

Our previous studies (Ranger JJ et al., unpublished data) have been reported that GFP-positive circulating positive tumor isolated from the blood exhibited a very low flow cytometry count. This is a consequence of the low efficiency of the transcriptional activity of the IRES bicistronic motif. In order to accomplish this task, we decided to established

primary circulating tumor cell culture from fresh blood collection. We still try optimize our cell culture conditions. However, using confocal microscopy technique and anti-GFP antibody, we detect a strong GFP-signal in blood samples from RCP-MTB-NDL treated with doxycycline (data not shown).

Objective 3. Training and Coursework in Cancer Pathobiology (Years 1-2 and 3)

UC Davis Extension Pathobiology of the Mouse Course tier-1IA.
The course was not available as a function of our schedule time. I will retry for the Year-3. Actually, for professional instance, Dr Cardiff referred me to his pathologist assistant Dr Lanu for any specimen examination.

3.2.2. Opportunities for training and professional development

Professional development-1.
Throughout supervision with research assistant and lab technician, I was trained and supervised to practice by myself mammary fat pad and tail vein injection assays. In the regards of my future career as an independent researcher, it appears to be highly useful to learn new lab techniques including in vivo assay using different mouse strains.

Professional development-2.
In collaboration with IRIC institute, Montreal, Canada, I was trained for mass spectrometry analysis as well as bioinformatics. I acquired solid expertise to be allowed me to develop my own research project based on proteomics study.

3.2.3. Dissemination of the results.
Nothing to Report.

3.2.4. Ongoing research for Year-3
Based on original **SOW**, here is a summary of research plan covering Year-3.

SOW-Task-1. During Year-3 I will respect and follow the ACURO animal appendix as fulfilled and officially approved for acceptance in the original award application. According to guidelines and regulations of Canadian Council on Animal Care I will complete the renewal of certification by January 1st of year-2016 coordinated with Ms Susanne Smith, Director of animal compliance office, McGill University.

Objective 2. Characterize mammary tumour progression as well as metastasis formation in RCP-proficient and RCP-deficient mouse model.

SOW-Task 5d. Cross TetO-NIC-RCP with RCP-MTB mice. Estimate 20 mice per group: wt and floxed RCP with doxycyclin and 10 mice per group: wt and floxed RCP without doxycycline = 60 mice in total.

Erratum: Here is NIC/RCP is not a TetO system and no breeding with RCP-MTB. The tumor onset experiment NIC/RCPKO includes: NIC/RCP-WT (n=20 mice), NIC/RCP-het (n=20 mice) and NIC/RCP-homo (n=20 mice) for a total of 60 mice.

Evolution: *The SOW-Task **5d** was completed at 100% during the present Year-2.*

SOW-Task 5e. Mammary gland outgrowth experiment using MMTV-IRES-Cre-floxed-RCP and MMTV-floxed-RCP. Mammary glands extraction, H&E

Evolution: The SOW-Task **5e** was completed at 100% during the present Year-2.

SOW-Task 5g and 5h. *Monitor tumor-onset of RCP-deficient erbB2 mice. Lung was collected for metastasis analysis. Estimate 20 mice per group. RCPwtNIC, RCPhomoNIC and RCPhetNIC= 60 per experiment. Data analysis and quantification of tumorigenesis and metastasis. Tumor onset experiment will be expressed as a Caplan-Meyer survival curve and lung metastasis will quantify as in Task-4, objective 4d.*

Evolution: SOW-Tasks **5g** and **5h** are complete at 90% and will be completed by next report.

Task 7. Role of RCP in intravasation and lodging of ErbB2 derived tumor (Years 1-2)

SOW-Task 7a. *Blood was collected from mice subjected for tumor onset experiment as in Obj.2 Task 2-1c at the time of sacrifice and GFP-positive tumoral cells were counted by flow cytometry. Estimate 20 mice per group = 40 mice in total*

During the Year-2 we optimize our initial approach to increase the yield of GFP-positive cells isolated from the bloodstream of our RCP-MTB-NDL mice. During Year-3 we plan to complete the enumeration of the number of GFP-positive tumor cells using fluorescence microscopy approach.

SOW-Task 7b. *Infect primary cells from the RCP-proficient and -deficient tumors with a mCherry lentiviral construct.*

During Year-3 we plan to established mCherry+/RCP-MTB-NDL(GFP+) cells and mCherry+/NIC/RCP cell lines. The lentiviral injection of a mCherry fluorescent protein will be useful to discriminate GFP- and GFP+ cells and therefore acts as an internal positive tracker during tail vein injection.

SOW-Task 7c. *Tail vein injection RCP-proficient and deficient tumors cells in immunocompromised mice. Animals will be sacrificed 4 weeks after cell injection. Lungs are collected and GFP (RCP-proficient) as well as mCherry-cells (RCP-proficient and deficient) are counted by flow-cytometry. Estimate 5 mice per group = 40 mice per experiment.*

During the Year-3, we plan to assess our established cell lines as in Task **7b** to metastasis study. Using flow cytometry, tail vein injection efficiency will be monitor by the detection of mCherry signal whereas the induction of RCP in NDL tumor cells will be confirm by the detection of GFP signal.

Objective 3. Training and Coursework in Cancer Pathobiology (Years 1-2 and 3)

UC Davis Extension Pathobiology of the Mouse Course tier-1B

IMPACT

Over the Year-2 of this research proposal, I improved the manuscript untitled: *Rab Coupling Protein (RCP) is a critical negative regulator in ErbB2-mediated mammary tumor progression* throughout multiple submission and revision processes. In order to increase the impact of this present research proposal in research community, we decided to divide in two separated stories the RCP project. Firstly, we plan to publish data related to the characterization of RCP in senescence induction in ErbB2 tumors (as mentioned in previous Report). Secondly, we already sent our RCP paper untitled: *Rab Coupling Protein (RCP) is a critical negative regulator in ErbB2-mediated mammary tumor progression* at editor board of Cancer Research journal. This latter paper highlights that RCP acts as tumor suppressor in ErbB2-driven model by negatively regulating both EMT and lung metastasis and bridges the cell junction dynamic feature and induction of senescence through the modulation of ZONAB, a transcriptional factor which is known to interacts with the tight junction marker ZO-1 and demonstrate abilities to translocate from cell surface into the nucleus to regulate the transcription of proliferation markers such as and *pcna.*
Finally, genomic human data sets highlights that expression of RCP is inversely correlated with Her2 molecular and clinical status where RCP can actually be used as a potent prognostic marker in Her2-positive breast cancer patient.

Impact on other disciplines

Nothing to report.

Impact on technology transfer.

Nothing to report.

Impact on society beyond science and technology.

Nothing to report.

Section: Changes / problems

Regarding Task **7a**, instead using the flow cytometry for detection of GFP-positive circulating tumor cells, we decided to change our approach to increase the yield of GFP signal. Using confocal microscopy, we are allowed now to quantify the number of GFP-tumor cells circulating throughout the bloodstream of RCP-MTB-NDL mice. This modification do not alter any original project outlines.

Changes that had a significant impact on expenditures

Nothing to report

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents

Nothing to report

Products

Nothing to report

Journal publications

Submitted in Cancer Research CAN-15-2782 , submission date 2015-10-06 12:05:59.

Untitled—*Rab Coupling Protein (RCP) is a critical negative regulator in ErbB2-mediated mammary tumor progression*

Authors—Pierre-Luc Boulay[1], Louise Mitchell[2], Jason Turpin[1], Julie-Émilie Huot-Marchand[1], Cynthia Lavoie[1], Virginie Sanguin-Gendreau[1], Laura Jones[1], Shreya Mitra[5], Julie M. Livingstone[3], Shirley Campbell[4], Michael Hallett[3], Gordon B Mills[5], Morag Park[1], Lewis Chodosh[6], Douglas Strathdee[2], Jim C. Norman[2] and William J. Muller[1]

Affiliations—*From [1]Department of Biochemistry, McGill University, Rosalind and Morris Goodman Cancer Research Montreal, QC, H3A 1A3, Canada, [2]Cancer Research UK Beaston Institute, Glasgow, G61 1BD,UK,[3] Department of Bioinformatics, McGill University, Rosalind and Morris Goodman Cancer, Montreal, QC, H3A 1A3, Canada, [4]Department of Pharmacology, University of Montreal, QC, H3C 3J7, Canada, [5]Department of System Biology, The University of Texas MD Anderson Cancer Center, Houston, Texas, 77030, USA, [6]Abramson Family Cancer Research Institute, University of Pennsylvania School of Medicine, Philadelphia, PA 19104, USA*

Books or other non-periodical, one-time publications

Nothing to report**.**

Other publications, conference papers, and presentations

Nothing to report.

Website(s) or other Internet site(s)

Nothing to report.

Technologies or techniques

Nothing to report.

Inventions, patent applications, and/or licenses

Nothing to report.

Other Products

Product-2. In order to complete the *in vivo* characterization of RCP in ErbB2-driven model, we generated during Year-1 with overlap with Year-2, a loss of function RCP model which consist in Cre recombinase system. To end this, we crossed mice bearing a conditional RCP allele with a MMV/ErbB2/IRES/Cre model.

PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Name:	**Louise Mitchell**
Project Role	Research assistant
Nearest person month worked	1 year
Contribution to Project	Ms Mitchell established the RCP knockout mouse model
Funding Support	Nothing to report

Name:	**Jason Turpin**
Project Role	PhD Student
Nearest person month worked	1 year
Contribution to Project	Mr Turpin established a MMTV-neuNDL cohort under doxycycline treatment (Internal control to support our original RCP-MTB-NDL doxycycline inducible model
Funding Support	Nothing to report

Name:	**Julie-Émilie Huot-Marchand**
Project Role	Research assistant
Nearest person month worked	1 month
Contribution to Project	Ms. Huot executed experimental procedures regarding immuno-staining on breast tumor tissue.

Funding Support	Nothing to report
Name:	**Cynthia Lavoie**
Project Role	Animal Health technician
Nearest person month worked	2 months
Contribution to Project	Ms. Lavoie practiced mammary fat pad injection of RCP/ErbB2 cell lines. Ms. Lavoie managed the floxed -RCP X FVB backcrosses, maintain ErbB2/NIC, ErbB2/neuNDL and MTB mouse strains.
Funding Support	Dr Muller CIHR Canadian grant
Name:	**Dongmei Zuo**
Project Role	Research associate
Nearest person month worked	0.5 month
Contribution to Project	Dr Zuo performed 3D cell culture using Gel Trek matrix.
Funding Support	Dr Muller CIHR Canadian grant
Name:	**Julie-Marie Livingstone**
Project Role	Bioinformatic expert
Nearest person month worked	0.5 month
Contribution to Project	Ms. Livingstone practiced bioinformatic analyses of clinical status of RCP using human genomic data sets.
Funding Support	Dr Hallet CIHR Canadian grant
Name:	**Shirley Campbell**
Project Role	Post-doctorate fellow
Nearest person month worked	1 month
Contribution to Project	Dr. Campbell accomplished cDNA cloning pTet-RCP-IRES-GFP
Funding Support	Dr Muller CIHR Canadian grant
Name:	**Dr. Gordon Mills**
Project Role	Professor
Nearest person month worked	0.5 month
Contribution to Project	Because of his expertise in terms of GTPases, Dr Mills, provide some key important input such as investigation of E-cadherin and Claudin-7 in our RCP-MTB-NDL breast tumor specimens.
Funding Support	MD-Anderson Cancer centre institute

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

Nothing to Report

What other organizations were involved as partners

Organization-1	
Organization Name	Beaston Research Institute
Location of Organization:	Glasgow, UK
Partner's contribution to the project:	Dr. Jim C. Norman, Louise Mitchell, Douglas Strathdee
Financial support:	Nothing to report
Facilities:	Nothing to report
Collaboration:	Provide us floxed-RCP frozen embryos
Organization-2	
Organization Name	MD Anderson Cancer Centre
Location of Organization:	Houston, TX, USA
Partner's contribution to the project:	Dr. Gordon Mills
Financial support:	Nothing to report
Facilities:	Nothing to report
Collaboration:	TCGA human data sets analyses
Organization-3	
Organization Name	Institute for Research in Immunology and Cancer, Montreal (IRIC), Canada.
Location of Organization:	Montréal, Canada
Partner's contribution to the project:	Dr. Eric Bonneil
Financial support:	Nothing to report
Facilities:	Proteomic
Collaboration:	Histone methylation profiling
Organization-4	
Organization Name	Bioinformatic department of McGill University
Location of Organization:	Montréal, Canada
Partner's contribution to the project:	Dr Mike Hallet / Julie-Marie Livingstone
Financial support:	Nothing to report
Facilities:	Bioinformatic
Collaboration:	Human gene expression profile
Organization-5	
Organization Name	University of Pennsylvania School of Medicine
Location of Organization:	Pennsylvania, USA
Partner's contribution to the project:	Lewis Chodosh
Financial support:	Nothing to report
Facilities:	Nothing to report
Collaboration:	MMTV-LTR-rtTA (MTB) mouse model

SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS: Nothing to report

QUAD CHARTS: Nothing to report.

Appendices

Materials and Methods

Antibodies and reagents— Rab11FIP1/RCP and β-Actin antibodies were from Sigma Aldrich. ZO-1 antibody were from Invitrogen Life Technologies. The PCNA antibody was from Millipore. All reagents used for IHC, IHF, and Western blotting were purchased from Biobasic, Bioshop and Sigma Aldrich respectively. Herceptin was purchased from Genetech. 7.16.4 murine antibody was produced in Hybridoma facility, McGill University.

Animals— An inducible RCP construct was engineered by cloning a His-RCP fragment (pcDNA3.1 cut with HindIII+Xba1) into a pTet-IRES-*GFP* plasmid using EcoRV restriction sites. The pTet-His-RCP-IRES-GFP DNA plasmid was linearized with PvuI and transgene integration was performed following standard techniques using the involving the microinjection of FVB one cell mouse embryos. Females were bred with male MTB (MMTV-rtTA) mice and treated with doxycycline to induce RCP expression within the mammary epithelium. To generate a conditional knockout allele for RCP/Rab11fip1 a targeting strategy was devised which flanked exon 2 of RCP (ENSMUSE00000210018; Ensembl Assembly GRCm38.p3, www.ensembl.org). To construct the gene targeting vector for RCP/Rab11fip1, a 129S7/SvEv bacterial artificial chromosome (BAC) for the genomic locus was initially identified on Ensembl. Three homology arms were sub-cloned from the BAC by recombineering in DY380 *E. coli* according to standard protocols. The arms were then cloned into the targeting vector pFlexNeoDTA, a modified version of pFlexible, with a PGK-EM7-Neo cassette (from pL452; replacing the puro*Δ*tk and the introduction of a DTA negative selection cassette (from pROSA26-1. The targeting vector was linearized by restriction digestion and electroporated into HM1 mouse embryonic stem cells and selected under G418 (240ug/ml active reagent). Surviving colonies were picked and screened for targeting by long-range PCR reactions using the Expand Long Template PCR System (Roche). Appropriate targeting was confirmed at both the 5' and 3' sides. The presence of the isolated loxP was confirmed by PCR across the site. Following identification of correctly targeted clones, mouse lines were generated by microinjection of ES cells into C57BL/6J blastocysts according to standard protocols. Following mating of chimeras, the germline offspring were identified by coat colour and the presence of the modified allele was confirmed with the 3' loxP primers. Mice were subsequently crossed with a mouse line ACTB::FLPe (C57B6/N background) delete the selectable marker by recombination at the FRT sites. All mice were housed in the animal facility of the Rosalind and Morris Goodman Cancer Center and all experiments were performed according to the animal care guidelines at the Animal Resource Centre of McGill. Once a week, mammary tumors were monitored via physical palpation and measurements were quantified using caliper measurement. Animals were sacrificed 8 weeks following initial palpation.

Histology—Mammary tumors and lung specimens were collected from tumor-bearing mice 8 weeks after initial palpation. Tissues were fixed overnight in 10% neutral buffered formalin, paraffin-embedded, and sectioned at 4 μm at the Histology Core Facility of Rosalind and Morris Goodman Cancer Centre. Tumor sections were stained with hematoxylin and eosin. In the lung metastasis studies, 5-step sections from a total depth of 50 μm for each lung sample were processed and assessed for metastatic lesion by hematoxylin and eosin staining. Lung metastases were counted manually using Scanscope microscope software.

DNA Plasmids and shRNAs— pcDNA3.0-His-RCP (a gift from Jim Norman) was cut with HindIII+Xba1 restriction enzymes and then the His-RCP insert was subcloned into pMSCV-hypgro (Clonetech) using Hpa1 sites to generate pMSCV-hygro-His-RCP. Small hairpin RNAs (shRNAs) control (shRNA-GFP) or against RCP (shRNA-RCP) were obtained from Open Biosystems.

Cell culture—ErbB2/neuNT (TM15-clone 6)-derived tumor cells were maintained in Dulbecco's Modified Eagle Medium supplemented with 10% FBS and SingleQuots (Clonetics). SKBR3 and BT474 human cell lines were maintained in Dulbecco's Modified Eagle Medium supplemented with 10% FBS. ErbB2/neuNDL and,ErbB2/neuNT (TM15 clone-6), SKBR3 and BT474 cell lines expressing shRNA-RCP or shRNA control were generated using 293T cells and lentivirally transduced according to the manufacturer's instructions followed by puromycin selection (4 μg/mL; Sigma-Aldrich). A similar subset of cell lines overexpressing pMSCV-hygro-His-RCP or pMSCV-control (empty vector) were generated using 293VSV cells retroviral transduced according to the manufacture's recommendations followed by hygromycin selection (500 μg/mL; Wisent).

Orthotopic transplants— For mammary fat pad transplants, 2.5×10^5 cells were injected into the inguinal mammary fat pad of athymic NCr mice and tumor measurements were performed twice a week. All animals were sacrificed and tumors were dissociated once the primary tumor volume reached 1500 mm^3. For tail vein injection, 2.5×10^5 cells were injected into caudal vein of athymic NCr mice and sacrificed after 4 weeks. Experimental metastasis assays were performed by injecting 5×10^5 cells directly into caudal tail vein of NCr athymic mice. All mice were sacrificed 4 weeks after injections, lungs were collected and processed to histology and then scoring of lung metastases was performed by enumeration of metastasis for each whole lung specimen.

*Immunoblotting and poly-ubiquitination assay- Frozen tissues and c*ells were processed in TGH buffer supplemented with protease inhibitors and 1 nM sodium orthovanadate. Tumor and cell lysates were solubilised at 4 °C for 30 min. Where indicated, tumors were subjected to cell fractionation as described [32]. Quantification of protein levels was performed using the Bradford protein assay. Eluted proteins were run on polyacrylamide gels and transferred onto polyvinilidene difluoride (PVDF) membranes. Protein expression level was detected using specific primary antibodies, secondary HRP-conjugated antibodies and chemiluminescence and detected using a developer. Quantification of protein levels was performed using ImageQuant 5.2 software.

S*tatistical Analysis*— Statistical analysis was performed using either a one-way or two-way ANOVA followed by a Bonferroni multiple comparison test as well as Student-T test using GraphPad Prism (version 4.0a, San Diego, CA).

Appendices

Legends

Figure 1. RCP regulates ErbB2-mediated tumor growth in NCr athymic mouse model— (A) Cell lines were infected with either an empty vector (EV), His-RCP (RCP) or shRCP, pretreated with IgG or 7.16.4 antibody and 2.5 x 10^5 cells were injected into the inguinal mammary fat pad of athymic mice. The tumor growth was monitored using caliper until single tumor reach end point guidelines (around a volume of 3 cm^3). (B) Cells were infected as in A, injected into the fat pad and monitored for tumor growth. Animal bearing a tumor of a volume of 0.5 cm^3 are subjected to IgG or 7.16.4 treatment (twice a week). Breast tumors are collected 8 weeks after the initial cell injection. Quantification of ErbB2/TM15 tumor growth is representative of five independent mice per experimental condition. Significance was determined by student t-test. *P<0.05 as compared to each paired control IgG condition.

Figure 2. RCP regulates ErbB2-driven lung metastasis— (A) Cell lines were infected with either an empty vector (EV), His-RCP (RCP) or shRCP, pretreated with IgG or 7.16.4 antibody and 5.0 x 10^5 cells were injected into the caudal tail vein of athymic mice. The lungs were collected 4 weeks post-injection and subjected to H&E.Quantification of ErbB2/neuNDL lung metastasis analyses is representative of four independent mice per experimental condition; H&E-stained lungs were scored for the number of metastases. Significance was determined by student t-test. ***P<0.001, *P<0.05 as compared to the empty vector (EV) control condition.

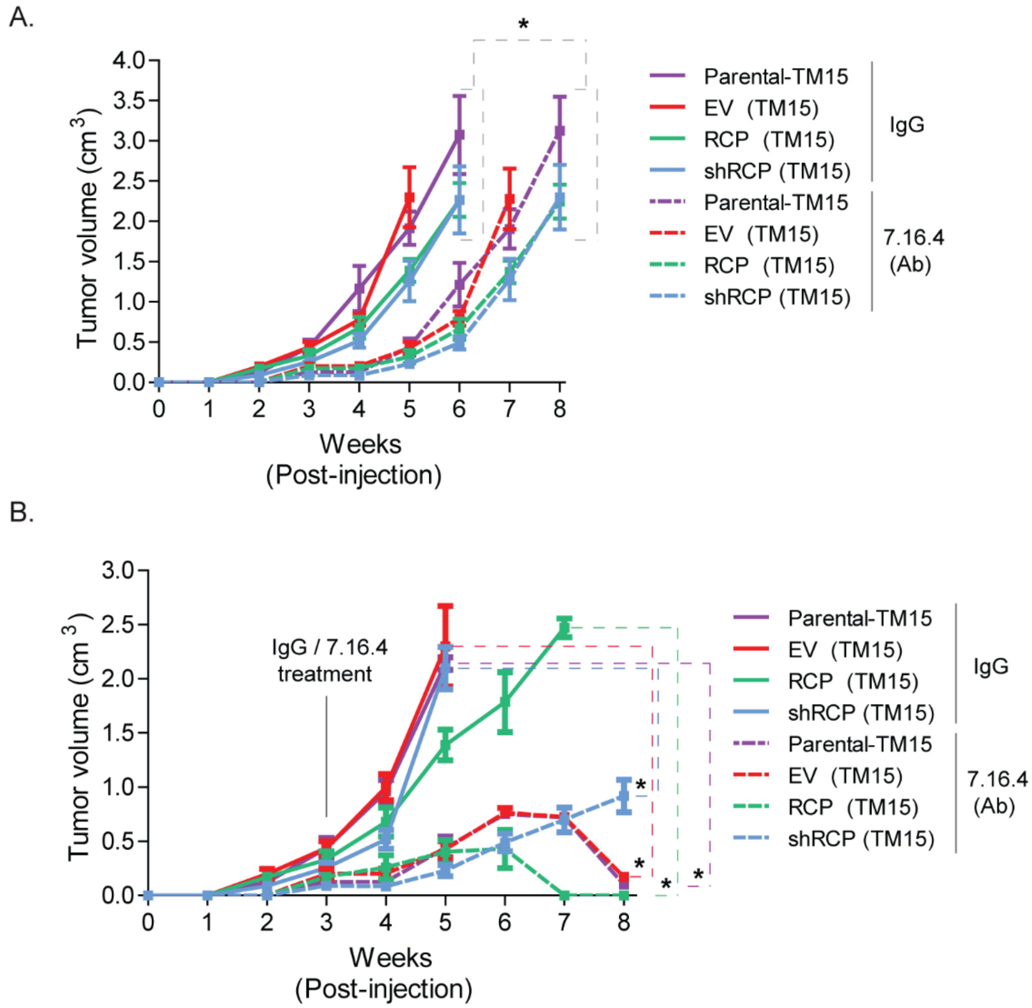
Figure 3. RCP is a negative regulator of ErbB2/neuNDL-driven mammary tumourigenesis.— **A.** Kaplan-Meier curve showing the tumor onset of NIC/RCP-WT, NIC/RCP-Het and NIC/RCP-Homo mice and analysis was performed as in A. The number of animals per experimental condition are; NIC/RCP-WT n=19, NIC/RCP-Het n=30, NIC/RCP-Homo n=35. **B.** Kaplan-Meier curve representing the tumor onset of RCP-MTB and RCP-MTB-NDL mice that were induced or not with dox from 12 weeks of age (n is the number of animals analyzed in each strain). Collected data were subjected to survival curve analysis and analysis of variance. The number of animals per experimental condition are; RCP-MTB without dox n=10, RCP-MTB with dox n=9, RCP-MTB-NDL without dox n=30. RCP-MTB-NDL/RCP-Low with dox n=12, RCP-MTB-NDL/RCP-High with dox n=15, NDL without dox n=11. Significance was determined by the student t-test. *P<0.05 are values compared to the cohort without dox. C**.** Sets of RCP-MTB-NDL tumors and cell lysates (positive-control, Con+ TM15 cells) were assessed by Western blot analysis for indicated proteins.

Figure 4. RCP regulates ErbB2-driven lung metastasis— Lung metastases were counted using Image Scope software. The number of lung metastases bearing animals (left panel) and the number of lung metastases per animal (middle panel) was quantified.. Quantification of the intra-vascular and extra-vascular lung metastases (right panel) was performed by analyzing H&E stained lung slides. The significance determined by the student t-test (left panel). Significance was determined using the student t-test, indicated p<0.05 are values compared to the control RCP-MTB-NDL without doxycycline (-DOX). Results represent the mean ± SEM of 12 non-induced and 12 induced animals.

Appendices

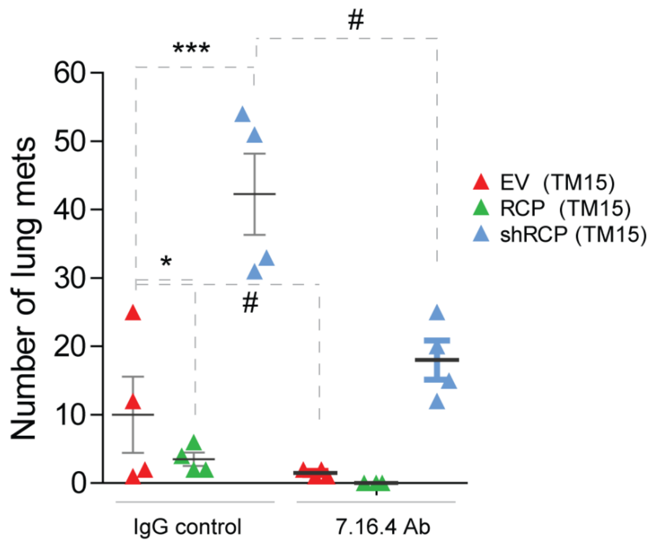
Figures

Figure 1. Boulay et al.



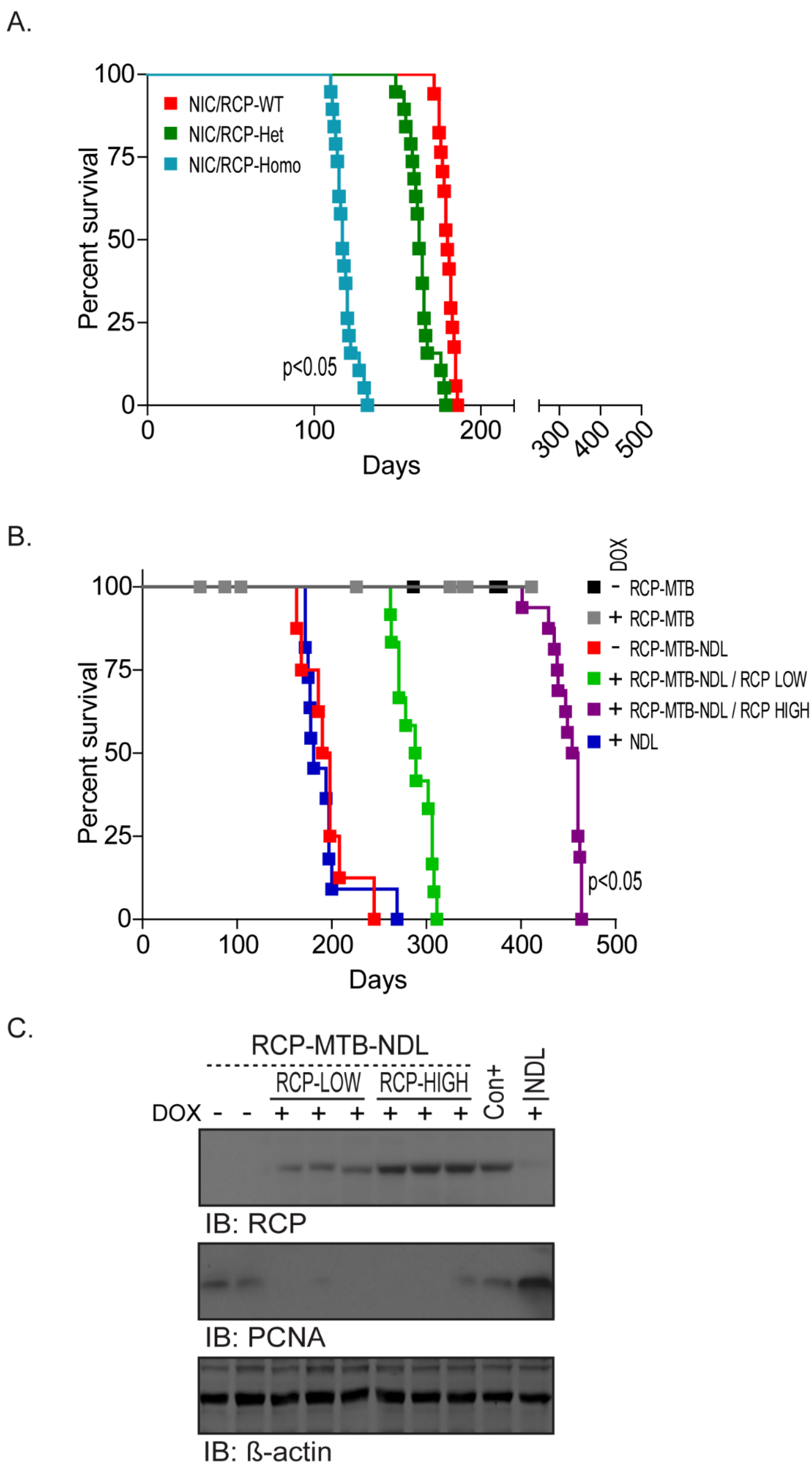
Figures

Figure 2., Boulay et al.



Figures

Figure 3., Boulay et al.



Figures

Figure 4., Boulay et al.